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Plastoquinol as a singlet oxygen scavenger in photosystem II

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Abstract

It has been found that in *Chlamydomonas reinhardtii* cells, under high-light stress, the level of reduced plastoquinone considerably increases while in the presence of pyrazolate, an inhibitor of plastoquinone and tocopherol biosynthesis, the content of reduced plastoquinone quickly decreases, similarly to α-tocopherol. In relation to chlorophyll, after 18 h of growth under low light with the inhibitor, the content of α-tocopherol was 22.2 mol/1000 mol chlorophyll and that of total plastoquinone (oxidized and reduced) was 19 mol/1000 mol chlorophyll, while after 2 h of high-light stress the corresponding amounts dropped to 6.4 and 6.2 mol/1000 mol chlorophyll for α-tocopherol and total plastoquinone, respectively. The degradation of both prenyllipids was partially reversed by diphenylamine, a singlet oxygen scavenger. It was concluded that plastoquinol, as well as α-tocopherol is decomposed under high-light stress as a result of a scavenging reaction of singlet oxygen generated in photosystem II. The levels of both α-tocopherol and of the reduced plastoquinone are not affected significantly in the absence of the inhibitor due to a high turnover rate of both prenyllipids, i.e., their degradation is compensated by fast biosynthesis. The calculated turnover rates under high-light conditions were twofold higher for total plastoquinone (0.23 nmol/h/ml of cell culture) than for α-tocopherol (0.11 nmol/h/ml). We have also found that the level of α-tocopherolquinone, an oxidation product of α-tocopherol, increases as the α-tocopherol is consumed. The same correlation was also observed for γ-tocopherol and its quinone form. Moreover, in the presence of pyrazolate under low-light growth conditions, the synthesis of plastoquinone-C, a hydroxylated plastoquinone derivative, was stimulated in contrast to plastoquinone, indicating for the first time a functional role for plastoquinone-C. The presented data also suggest that the two plastoquinones may have different biosynthetic pathways in *C. reinhardtii*.

Keywords: Photosystem II; Plastoquinol; Plastoquinone; Pyrazolate; Singlet oxygen; Tocopherol; Tocopherolquinone

1. Introduction

Tocopherols are a group of lipophilic antioxidants synthesized exclusively by photosynthetic organisms, occurring mainly in leaves and seeds [1]. Their antioxidant function is attributed to the inhibition of membrane lipid peroxidation and scavenging of reactive oxygen species [1–3] but also other functions have been shown in plant metabolism such as role in sugar export from leaves to phloem [4].

Tocopherols are also important antioxidants in human metabolism and it was also recently suggested that these lipids may have some function in the regulation of genes expression [5,6].

We have recently proposed that the specific function of α-tocopherol is the protection of photosystem II (PSII) against singlet oxygen formed by the reaction center triplet chlorophyll in *Chlamydomonas reinhardtii* [2,3]. Tocopherol scavenges singlet oxygen and prevents degradation of D1 and D2 reaction center proteins and the loss of photosynthetic activity [2,3]. In scavenging of singlet oxygen, α-tocopherol is irreversibly oxidized and its continuous resynthesis takes place to keep its

Abbreviations: Chl, chlorophyll; DPA, diphenylamine; HBA, *p*-hydroxybenzoic acid; HL, high light; HGA, homogentisic acid; LL, low light; NPQ, nonphotochemical quenching; PSII, photosystem II; PQH_2, plastoquinol; PQ, plastoquinone, pyr, pyrazolate; TLC, thin-layer chromatography; Toc, Tocopherol; TQ, tocopherolquinone; UQ, ubiquinone

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level sufficient for the photoprotection. It has been also shown in *Arabidopsis thaliana* that tocopherol plays a specific function in the maintenance of photosystem II function [7] and that this function supplements the photoprotective function of nonphotochemical quenching (NPQ). While the NPQ-deficient *npq4* mutant and the tocopherol cyclase-deficient *vte1* mutant exhibited a rather moderate sensitivity to photosystem II inhibition in high light, dramatic loss of photosystem II activity was observed in high light in the *vte1 npq4* double mutant deficient in both NPQ and tocopherol. Moreover, it was found [8] that *npq4 A. thaliana* mutant alone, as well as mutant lacking additionally zeaxanthin (*npq1*) and lutein (*lut2*), had in high light ~50% higher α-tocopherol levels than the wild-type and ~7-fold higher α-tocopherol levels than low-light-grown plants. This indicates that the two photoprotective mechanisms cooperate and the increased α-tocopherol levels compensate for the lack of NPQ and xanthophylls.

It was shown that α-tocopherol is oxidized by singlet oxygen to α-tocopherolquinone *in vitro* [1,9,10] and an indication that such a reaction may proceed *in vivo* is the widespread occurrence of α-tocopherolquinone in plants as a minor leaf component [11], and its increased level under stress conditions [12–14]. Moreover, in addition to being produced in tocopherol turnover, also additional functions of α-tocopherolquinone have been suggested, such as protection of PSII against photoinhibition [15–17] and antioxidant action of the reduced form of this prenyllipid [18–26]. It has been previously demonstrated that during high-light illumination of *C. reinhardtii* cultures, grown in the presence of tocopherols and plastoquinone biosynthesis inhibitors, the addition of cell wall permeable short-chain tocopherols analogues and plastoquinone homologues rescued the photosynthetic activity and D1 and D2 protein degradation [3]. These observations suggested that also plastoquinones, especially in the reduced form, might have photoprotective action on PSII and may take the role of tocopherol in singlet oxygen detoxification. The reduced plastoquinone, as a phenolic compound is a potent singlet oxygen quencher and the antioxidant activity of plastoquinol has been already shown in several studies [21–23, 27].

Apart from the plastoquinone (PQ, PQ-9, PQ-A), whose role in the photosynthetic electron transport is well characterized, other plastoquinones, like plastoquinone-B (PQ-B) and plastoquinone-C (PQ-C) were isolated in the 60s by column and thin-layer chromatography (TLC) [28,29]. However, later it was suggested that these plastoquinones were isolation artifacts rather than of natural origin [30,31]. Using HPLC techniques, it was shown that these compounds are natural components of leaves and it was found that PQ-C, present mostly in the reduced form, accounts for 30% and 18.5% of PQ-A in spinach and maple leaves, respectively [32]. The structure of PQ-C was found to be a hydroxyl derivative of PQ-A with the hydroxyl group in the side chain and PQ-B to be a fatty acid ester of PQ-C [33,34]. In the reconstitution experiments [34] it was found that both PQ-C may substitute for PQ-A as an electron acceptor from photosystem II but its *in vivo* function and biosynthesis remains unknown.

In the present studies, we analyzed the content of both oxidized and reduced forms of plastoquinone together with that of tocopherols under high-light conditions in *C. reinhardtii* cultures, as well as the content of tocopherolquinones as possible oxidation products of tocopherols. The turnover of the prenyllipids was also followed by their ^{14}C-labeling and autoradiography of the chromatographically separated cell extracts.

2. Materials and methods

C. reinhardii was grown photoautotrophically at 25 °C under low-light (70 μmol m^2 s^{-1}) conditions in HS (high-salt) medium and bubbled with 5% carbon dioxide in air as described in [35]. The HS medium contained in 1 l the following nutrients: 1.44 g K_2HPO_4, 0.72 g KH_2PO_4, 0.5 g NH_4Cl, 0.02 g $MgSO_4 \cdot 7H_2O$, 0.01 g $CaCl_2 \cdot 2H_2O$, 50 mg EDTA, 22 mg $ZnSO_4 \cdot 7H_2O$, 11.4 mg H_3BO_4, 5.06 mg $MnCl_2 \cdot 4H_2O$, 4.98 mg $FeSO_4 \cdot 7H_2O$, 1.61 mg $CoCl_2 \cdot 6H_2O$, 1.57 mg $CuSO_4 \cdot 5H_2O$, 1.10 mg $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$. The experiments were started when cultures showed chlorophyll (Chl) concentration of 6 μg/ml. Pyrazolate (nonhydrolyzed, obtained from Wako Pure Chemicals, Neuss, Germany), an inhibitor of *p*-hydroxyphenylpyruvate dioxygenase [36], was added from 5 mM stock solution in DMSO (spectral grade, Merck) at the start of the growing time. Pyrazolate solution was stored in freezer. For the described experiments, 30 ml of cell culture was grown in 50 ml tissue culture bottles with CO_2 bubbling and additional continuous magnetic stirring to enable homogeneous cells distribution. The cultures were grown for 18–24 h under low light and additionally under high light (1500 μmol·m^2·s^{-1}) for up to 2 h. For Chl determination, 2 ml of the culture was taken and centrifuged for 30 s in an Eppendorf tube using a benchtop centrifuge (10,000 ×*g*), and the pellet of cells was extracted with 2 ml of acetone. Chl concentration was determined spectrophotometrically according to [37]. For HPLC analysis, 2 ml of the culture was centrifuged as above, the pellet was extracted with 0.5 ml of acetone, evaporated in a stream of nitrogen, dissolved in 200 μl of the HPLC-solvent, shortly centrifuged to remove undissolved material, and analyzed by HPLC.

2.1. HPLC analysis

Tocopherol (Toc) standards were purchased from Merck (No. 15496, HPLC-grade) and their concentrations were determined spectrophotometrically using ε_{292}=3.26 mM^{-1} cm^{-1} for α-Toc and ε_{298}=3.81 mM^{-1} cm^{-1} for γ-Toc in absolute ethanol [38]. The oxidized and reduced PQ standards were obtained as described in [39] and their concentrations determined using ε_{255}=17.94 mM^{-1} cm^{-1} for PQ and ε_{290}=3.39 mM^{-1} cm^{-1} for PQH_2 both in absolute ethanol [40]. Both α- and γ-tocopherolquinones (TQs) were obtained by oxidation of the corresponding tocopherols dissolved in methanol with 50% aqueous $FeCl_3$ and purified by column chromatography [39]. The ε coefficients of α-TQ and γ-TQ in ethanol used for quantification were taken as 18.83 mM^{-1} cm^{-1} at 268 nm [40] and 18.61 at 258 nm mM^{-1} cm^{-1} [41], respectively. The standard of PQ-C was obtained from maple leaves as described previously [32]. The ε coefficients of PQ-C was taken as that of PQ.

The HPLC measurements were performed with an injection volume of 100 μl using Jasco pump PU-980 and UV–VIS detector system UV-970, a Shimadzu RF10-AXL fluorescence detector (excitation/emission detection at 290/330 nm), a Teknokroma (Barcelona, Spain) C_{18} reverse-phase column (Nucleosil 100, 250×4 mm, 5 μm). Tocopherols, as well as oxidized and reduced plastoquinone were separated in methanol/hexane (340/20, v/v) at the flow rate of 1.5 ml/min and analyzed simultaneously during the same run [42].

TQs and PQ-C were determined using post-column reduction with Zn-column and fluorescence detection of the reduced prenylquinones according to [43]. The solvent was methanol, containing 10 mM $ZnCl_2$, 5 mM Na-acetate, and 5 mM acetic acid at the flow rate of 1 ml/min.

Ubiquinone-9 (UQ-9), obtained as a gift from Hoffman La Roche (Basel, Switzerland), was quantified from HPLC-chromatograms in the same solvent system as for tocopherols and plastoquinones but using absorption detection at 275 nm and ε_{275}=14.40 mM^{-1} cm^{-1} in ethanol [38] for standard solution preparation.

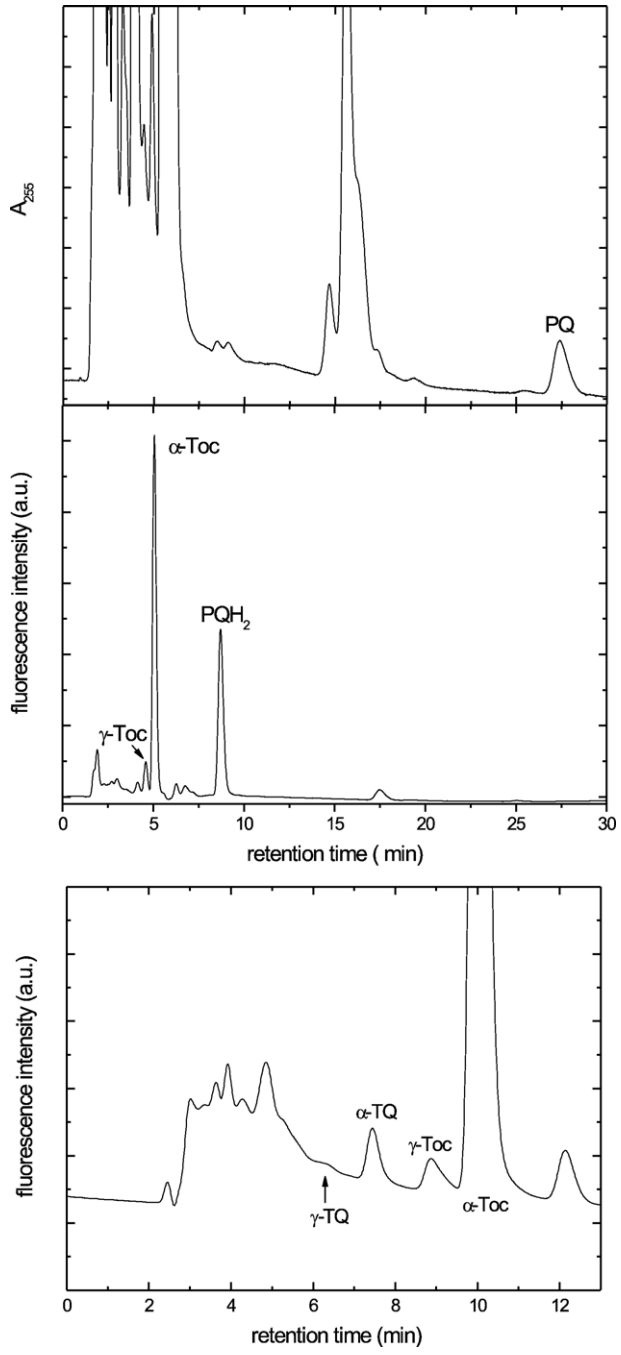

Fig. 1. HPLC chromatogram of an extract from *Chlamydomonas reinhardtii* cultures (grown for 18 h under low-light conditions) using simultaneous absorption detection at 255 nm (top) and fluorescence detection (excitation/emission — 290/330 nm) (center) on C_{18} reverse-phase column in methanol/hexane (340/20, v/v). Tocopherolquinones were detected using post-column reduction with Zn-column and fluorescence detection (excitation/emission — 290/330 nm) on C_{18} reverse-phase column (bottom). PQH_2 — plastoquinol, PQ — plastoquinone, Toc — tocopherol, TQ — tocopherolquinone. Further details are given in Materials and methods.

2.2. Determination of the photosynthetic activity

The oxygen evolution activity of *Chlamydomonas* cells was measured in the growing medium with the oxygen Clark-type electrode (Hansatech, England) using 1 ml sample volume at 25°C, Chl concentration given in the figure and light intensity of 1500 μmol m^2 s^{-1}. Total oxygen evolution activity was measured on the whole cells without any artificial electron acceptors, while photosystem II activity was measured in the presence of 0.5 mM *p*-benzoquinone.

2.3. ^{14}C-labeling experiments

Homogentisic acid–U–^{14}C was prepared from radioactive L-tyrosine–U–^{14}C (20 μCi, 5.5 mCi/mmol) by dilution of the labeled L-tyrosine (MP Biomedicals) (495 mCi/mmol) with nonlabeled L-tyrosine (Sigma) by incubation with L-amino acid oxidase (from *Crotalus adamanteus* venom, type IV, Sigma) and afterwards with the α,α′-bipyridyl-inhibited rat liver homogenate according to [44]. The purity of the homogentisic acid was confirmed by thin-layer chromatography on silica gel developed in benzene–methanol–glacial acetic acid (25/25/4, v/v/v) [44] and it was found to be free from the labeled tyrosine. For incorporation experiments, 1.63 ml of the ^{14}C–homogentisic acid stock solution (0.92 mM, ~5 μCi/ml) was added to 30 ml of cell culture (final homogentisic acid conc. — 50 μM) together with 5 μM pyrazolate at the start of the growing time under low-light conditions for 18 h and 7.5 ml samples were taken during the subsequent high-light treatment, centrifuged, extracted with acetone, concentrated and spotted on silica gel TLC plate. Alternatively, the centrifuged cells were lyophilized, extracted overnight with hexane and spotted on TLC plate. The plates were developed in chloroform, exposed to Kodak phosphor screen for 3 days and scanned with phosphoimager.

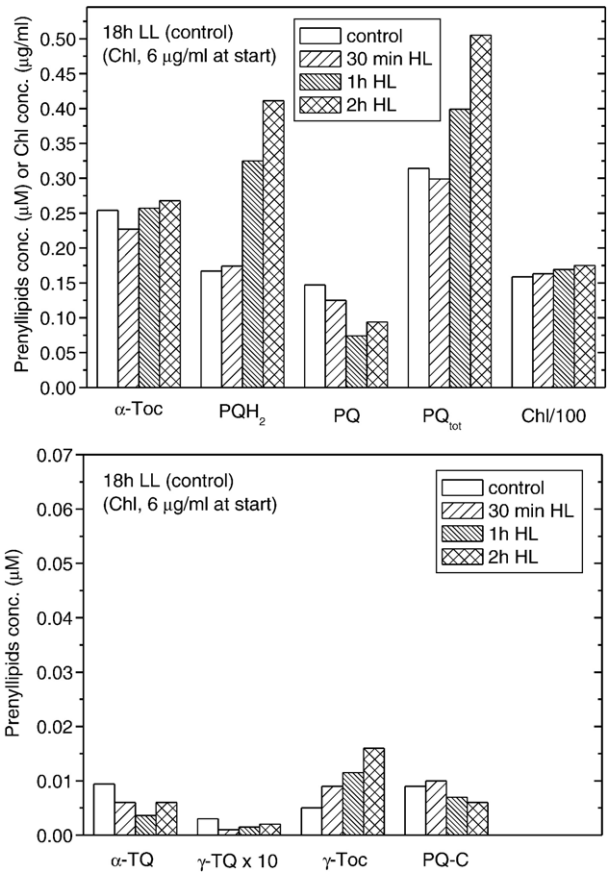

Fig. 2. The effect of high light on tocopherols, tocopherolquinones, plastoquinones and chlorophyll content in *Chlamydomonas reinhardtii* cultures. Chl — chlorophyll, LL — low light, HL — high light, PQH_2 — plastoquinol, PQ — plastoquinone, $PQ_{tot}=PQH_2+PQ$, Toc — tocopherol, TQ — tocopherolquinone. The errors (SD, $n\geq 3$) were ≤4% of the shown values for prenyllipids and ≤0.3% for Chl in the upper panel, and ≤7% in the lower panel. Other details are given in Materials and methods.

3. Results

For the analysis of both oxidized and reduced plastoquinone, together with tocopherols in one HPLC run, we applied simultaneous UV absorption detection of plastoquinone and fluorescence detection of tocopherols and plastoquinol (Fig. 1) taking advantage of their native fluorescence [45,46]. Tocopherolquinones were detected with high sensitivity after their reduction with a zinc-column to the fluorescent reduced forms (Fig. 1). When *C. reinhardii* culture grown for 18 h under low-light conditions was exposed to high light for 2 h, a slight increase in α-tocopherol and chlorophyll is observed, as well as twofold increase in plastoquinol content (Fig. 2 top, Table 1). The increase in plastoquinol level could be only partially at the expense of the oxidized form, and most of the increase must be due to de novo synthesis of plastoquinol under high-light conditions which manifests in the increase of total plastoquinone (Fig. 2 top) after 1 h of illumination or longer. This might suggest an important function of plastoquinol in response to high-light stress. Among other prenyllipids investigated, γ-tocopherol, an α-tocopherol biosynthetic precursor, was found to increase gradually during high-light treatment. It occurred only at 2% of the α-tocopherol amount in non-stressed cells (Table 1). Both α- and γ-tocopherolquinones, the possible oxidation products of the corresponding tocopherols, were identified in minor amounts but their level did not change significantly during strong light treatment. In contrast to plastoquinone, the content of its hydroxyl derivative, PQ-C, did not increase during the experiment.

In order to investigate the turnover rate of the prenyllipids under high light, the analogical experiments were performed in the presence of low concentrations of pyrazolate, an inhibitor of tocopherols and plastoquinone synthesis. At the concentrations of the inhibitor applied, the plastoquinone synthesis is only partially inhibited during the growth under low light so that the photosynthetic electron transport is not affected [2]. It can be observed that during high-light illumination, the content of α-tocopherol level gradually decreases (Fig. 3 top) in contrast to the control culture without the inhibitor (Fig. 2 top). Surprisingly, also the loss of both the reduced and oxidized forms of plastoquinone can be observed (Fig. 3 top, Table 1) during high-light treatment. The initial increase in plastoquinol level after 15 min of high-light illumination is probably due to reduction of plastoquinone in the electron transport chain. When the total plastoquinone content is compared with the α-tocopherol level during the light stress, a similar time dependence can be found. This dependence is opposite to the high-light effect observed in the control culture in the absence of the inhibitor (Fig. 2 top). The observed plastoquinone(ol) degradation under high-light stress, in the presence of the inhibitor has not been reported before and indicates that there is a high resynthesis rate of plastoquinol, even higher than that of α-tocopherol, under high-light conditions. The calculated turnover rates under high light [2], based on the data in Figs. 2 and 3, were 0.1 nmol/h for α-tocopherol and 0.22 nmol/h per ml of cell culture for total PQ (oxidized and reduced). In the presence of the inhibitor, the decrease in the content of both prenyllipids was also accompanied by gradual chlorophyll loss (Fig. 3 top). The decrease in γ-tocopherol was also observed. Together with the decrease in tocopherols level, an increase in the content of the corresponding tocopherolquinones, the oxidation products of

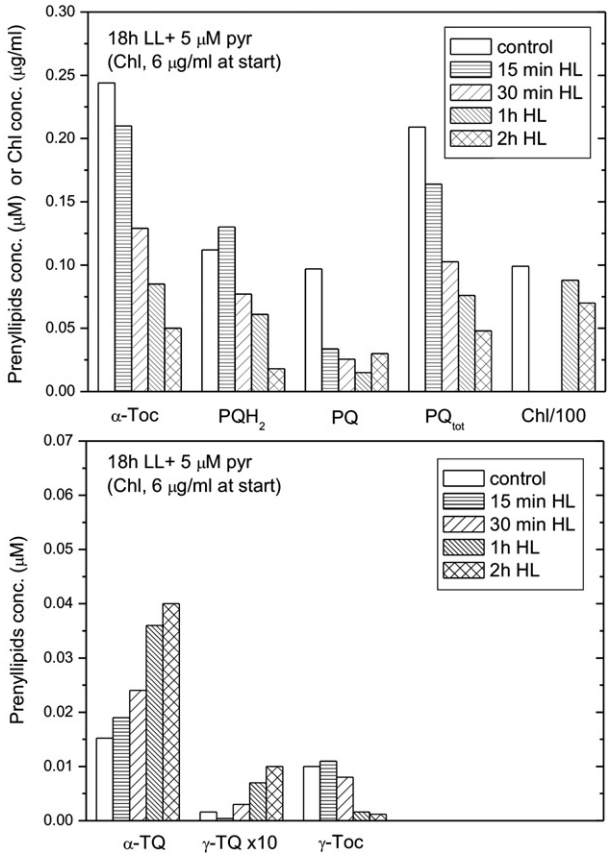

Fig. 3. The effect of high light on tocopherols, tocopherolquinones plastoquinone and chlorophyll content in *Chlamydomonas reinhardtii* cultures in the presence of 5 μM pyrazolate (pyr). The errors (SD, $n \geq 3$) were ≤6% of the shown values for prenyllipids and ≤0.3% for Chl in the upper panel, and ≤9% in the lower panel. Other details are given in Fig. 2 and Materials and methods.

Table 1
Influence of HL treatment and pyrazolate on prenyllipids content (mol/1000 mol Chl) of *Chlamydomonas reinhardii* cells

Prenyllipid	Prenyllipids content (mol/1000 mol Chl)			
	Control		5 μM pyrazolate	
	18 h LL	+2 h HL	18 h LL	+2 h HL
PQH_2	9.5	21.1	10.2	2.3
PQ	8.3	4.8	8.8	3.9
PQ_{tot}	17.8	25.9	19	6.2
α-Toc	14.4	13.8	22.2	6.4
α-TQ	0.5	0.3	1.4	5.1
γ-Toc	0.3	0.8	0.9	0.15
γ-TQ	0.02	0.01	0.014	0.13

LL — low light (70 μE/m^2/s), HL — high light (1500 μE/m^2/s), Chl — chlorophyll, PQH_2 — plastoquinol, PQ — plastoquinone, $PQ_{tot}=PQH_2+PQ$, Toc — tocopherol, TQ — tocopherolquinone. The errors (SD, $n \geq 3$) were ≤9% of the shown values.

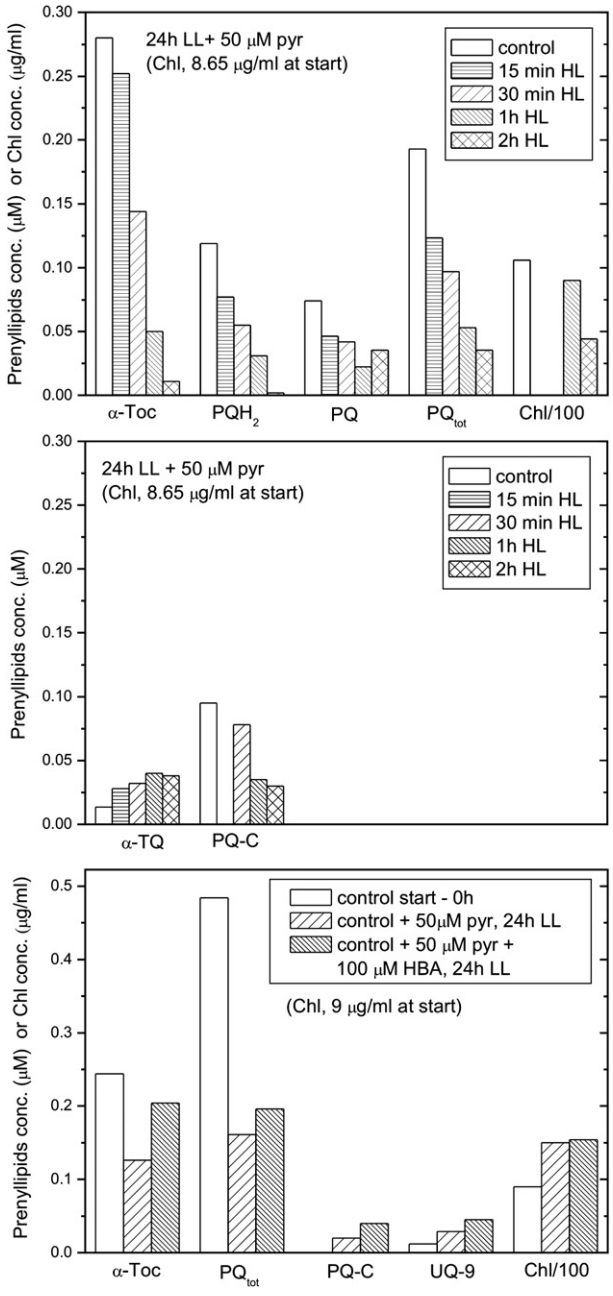

Fig. 4. The effect of high light on α-tocopherol, plastoquinone and chlorophyll (top), α-tocopherolquinone and plastoquinone-C (center) content in *Chlamydomonas reinhardtii* cultures in the presence of 50 μM pyrazolate. The effect of *p*-hydroxybenzoic acid (HBA) on the prenyllipids level in pyrazolate-inhibited *C. reinhardtii* cultures under low light (bottom). UQ-9 — ubiquinone-9. The errors (SD, $n \geq 3$) were ≤10% of the shown values for prenyllipids and ≤0.7% for Chl in the upper panel, ≤8% in the central panel. In the lower panel the errors were ≤9% for prenyllipids and ≤0.5% for Chl. Other details are given in Fig. 2 and Materials and methods.

tocopherols, was observed. After 2 h of high-light exposure, α-tocopherolquinone level approximates that of α-tocopherol but it corresponds only to about 25% of the initial α-tocopherol concentration. When the higher inhibitor concentration was applied (Fig. 4 top), the consumption of α-tocopherol and plastoquinol, as well as chlorophyll loss in high light was more pronounced, especially at the longer illumination time. This indicates that at 5 μM pyrazolate concentration, the prenyllipids synthesis was only partially inhibited.

The most unexpected effect of pyrazolate was on plastoquinone-C (PQ-C) level. It increased in control cultures (low light grown) tenfold in the presence of the inhibitor and amounted to 50% of the total plastoquinone level (Fig. 4 center), while its level accounted only for 3% in the absence of the inhibitor (Fig. 2 bottom). Under high-light illumination, the PQ-C level decreased gradually with time (Fig. 4 center) indicating that it is not an oxidation product of plastoquinone. The stimulatory effect of pyrazolate on PQ-C synthesis may suggest that its biosynthetic pathway is independent on the homogentisic pathway, common for tocopherols and plastoquinone, and the only known for these prenyllipids so far. The lack of the genes for this pathway in some cyanobacteria (e.g. *Synechococcus elongatus* 7942, *Thermosynechococcus elongatus* BP-1, *Prochlorococcus marinus* [47]) together with the mutant studies of the *Synechocystis* 6803 [48] and the presence of some ubiquinone (UQ) synthesis genes in this cyanobacterium may suggest that plastoquinone in cyanobacteria, as well as PQ-C in *C. reinhardtii*, is synthesized via similar

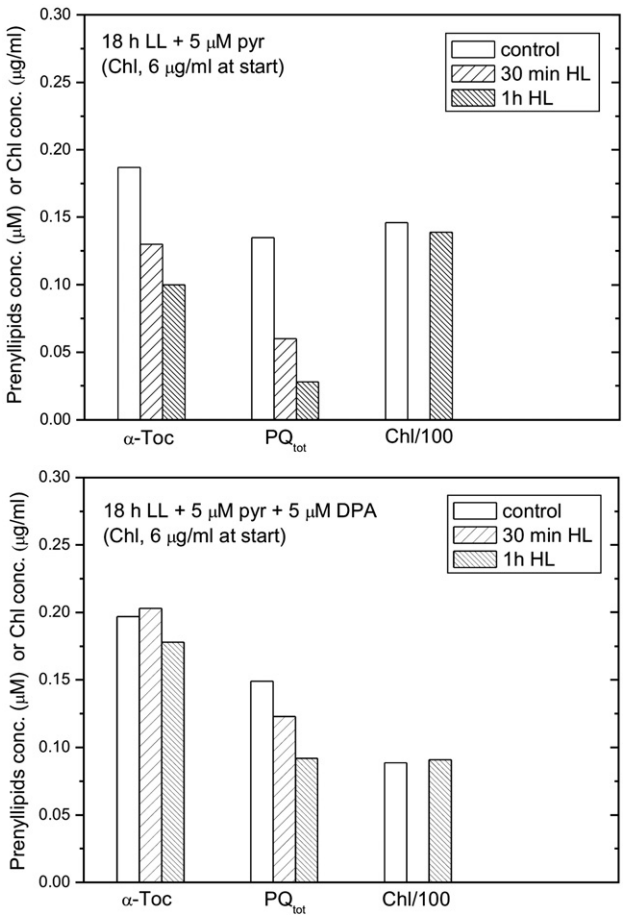

Fig. 5. Reversal of the effect of 5 μM pyrazolate on α-tocopherol and plastoquinone content under high light by the singlet oxygen quencher — diphenylamine (DPA). The errors (SD, $n \geq 3$) were ≤7% of the shown values for prenyllipids and ≤0.4% for Chl. Other details are given in Fig. 2 and Materials and methods.

intermediates as in ubiquinone biosynthesis pathway. Therefore, we tested the effect of the ubiquinone biosynthetic precursor, *p*-hydroxybenzoic acid, on PQ-C level in the presence of pyrazolate. As can be seen in Fig. 4 (bottom), *p*-hydroxybenzoic acid stimulated PQ-C synthesis twofold, while UQ synthesis increased by 50% during the same time of growth under low light. These results may suggest that PQ-C is synthesized at least partially by common intermediates with ubiquinone in *C. reinhardtii*. Interestingly, also α-tocopherol, as well as plastoquinone content to some degree was stimulated.

In order to verify if singlet oxygen is responsible for plastoquinone consumption during high-light stress, similarly as it was shown for tocopherol [2,3], we checked for the effect of diphenylamine, a singlet oxygen quencher [49], on the investigated reaction. It was found that diphenylamine inhibits both tocopherol and total plastoquinone uptake in the presence of pyrazolate (Fig. 5), suggesting that singlet oxygen might be responsible for both tocopherol and plastoquinone degradation.

It was shown [2] that in the presence of the inhibitor, tocopherol oxidation is accompanied by D1 protein degradation and this reduces PSII activity. In the case of plastoquinone, it is possible that its degradation affects also the efficiency of the total photosynthetic electron transport rate due to decrease in size of the PQ-pool. However, as shown in Fig. 6, total electron transport rate (PS) and PSII activity are affected to the same extent, indicating that decrease in the PQ-pool is not the limiting factor of the oxygen evolution inhibition in the investigated system.

In order to follow the radioactivity of the consumed tocopherol and plastoquinone during high-light treatment, we applied ^{14}C-labeled homogentisic acid, as the precursor of both prenyllipids, together with the pyrazolate to the cell culture and after a given time of high-light treatment the samples were extracted and separated by TLC and the radioactivity measured by autoradiography. Since both homogentisic acid and pyrazolate penetrate slowly into *C. reinhardtii* cells, the pulse-chase experiments were not possible. Therefore, both of these compounds were added at the start of the culture growth, i.e. 18 h before the strong light illumination. It can be found on autoradiograms (Fig. 7A and B) that the most labeled compound is plastoquinone and its level gradually decreases with the high-light illumination time. On the other hand, during 2 h of low-light growth (hexane extract), no significant decrease in the level of labeled PQ is observed. When the levels of both α-tocopherol and plastoquinone were analyzed by HPLC during high-light illumination in the presence of homogentisic acid and pyrazolate, their consumption was prevented (data not shown), similarly as it was previously demonstrated for α-tocopherol [2]. Thus, combining the results shown in autoradiograms with those of HPLC measurements obtained under analogical conditions, it can be concluded that the turnover of PQ is high under high light and low under low light. We have not identified the band corresponding to plastoquinol on TLC plates, but this is due its oxidation to plastoquinone during the sample preparation and TLC procedure. Therefore, the plastoquinone band observed on TLC plates corresponds to total plastoquinone. In the hexane extract, also labeled α-tocopherol was identified on TLC plate, that is quickly degraded after 1 h of high-light illumination (Fig. 7B). The intensity of the band corresponding to α-tocopherol is considerably lower than that of plastoquinone probably because of the lower homogentisic acid incorporation into tocopherol than into plastoquinone.

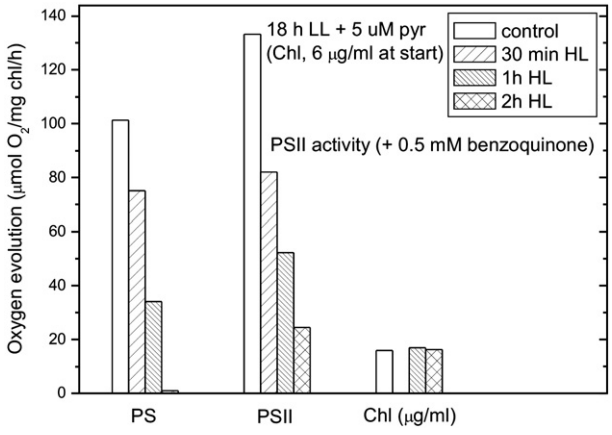

Fig. 6. Inhibition of total oxygen evolution activity (PS) and photosystem II activity (PSII) by 5 μM pyrazolate under high light. The activity was determined on whole cells in the absence of any artificial electron acceptors (PS) or presence of 0.5 mM *p*-benzoquinone (PSII). The errors (SD, $n \geq 3$) were ≤7% of the shown values for prenyllipids and ≤0.2% for Chl. Other details are given in Fig. 2 and Materials and methods.

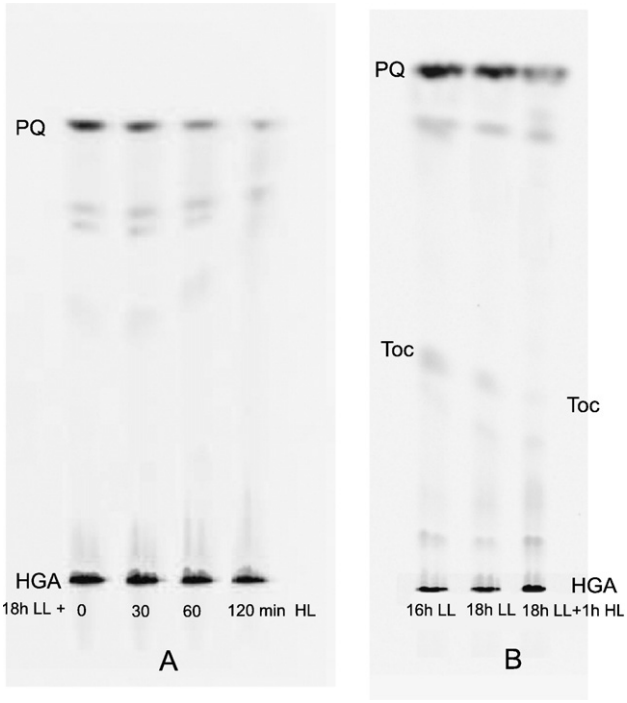

Fig. 7. Autoradiograph of TLC plate of an acetone (A) and hexane (B) extract of *Chlamydomonas reinhardtii* cells after exposure to 50 μM ^{14}C–homogentisic acid (HGA) together with 5 μM pyrazolate and high-light treatment. Other details are given in Materials and methods.

4. Discussion

It is well established that singlet oxygen is formed in photosystem II in the process of quenching of triplet state of the

reaction center chlorophyll P680 [50–55] and that the formed singlet oxygen induces degradation of the D1 protein [35,56–58]. The lost D1 protein is resynthesized and reassembled into a new functional photosystem II [59,60]. This turnover keeps photosystem II active in spite of singlet oxygen damage, as long as the expression rate of the D1 protein compensates for its degradation rate. However, under high-light stress this compensation may not be sufficient and, as a consequence, the entire photosystem II is degraded and photosynthesis ceases [59,60]. We have recently proposed that α-tocopherol is an efficient quencher of singlet oxygen formed by PSII in *C. reinhardtii* [2,3]. During scavenging of singlet oxygen, tocopherol is oxidized and irreversibly consumed. Continuous resynthesis of α-tocopherol is required to keep its concentration sufficient for PSII protection, thus high turnover rate of the tocopherol pool takes place.

In the present studies we have presented evidence that apart from tocopherol, also plastoquinol undergoes high turnover rate under high-light conditions and it leads us to the assumption that this is due to scavenging of singlet oxygen by plastoquinol *in vivo*. The data obtained previously [3], indicate that synthetic, cell wall permeable short-chain plastoquinones rescued photosynthetic activity in the presence of the inhibitor, indicating similar photoprotective mechanism of action of plastoquinones as that of tocopherol. Although the short-chain plastoquinones were applied in the oxidized form, it is known that benzoquinones are efficient electron acceptors from photosystem II, commonly used for measuring photosystem II activity, and they are readily reduced to the quinol forms. It was already demonstrated in many studies that plastoquinol, as a phenolic compound, shows antioxidant properties even stronger than tocopherol [21–23,27]. A physiological effect related to the antioxidant properties of both plastoquinol and α-tocopherol *in vivo* could be accumulation of these prenyllipids in tree leaves in plastoglobuli during the vegetation period, especially at high irradiance conditions [61]. α-Tocopherol was shown to be an efficient singlet oxygen scavenger in organic solvents and it was most active among the four isomers [1,9,10]. There are no literature data on that subject for plastoquinol, but we have found that it shows similar singlet oxygen quenching activity to α-tocopherol, while plastoquinone is a poor quencher (unpublished results). In contrast to tocopherol, which is irreversibly oxidized by singlet oxygen, plastoquinol is probably primarily oxidized to plastoquinone that can be at least in part regenerated to plastoquinol by PSII and it is recycled this way.

It should be mentioned that under high-light conditions also oxidation of α-tocopherol and plastoquinol by superoxide anion radical generated in photosystem I could be partially responsible for their consumption. This radical is produced within thylakoid membranes and it can be scavenged there by both prenyllipids [62,63].

It remains also to be examined why in the absence of the inhibitor under high light, when the tocopherol turnover should be fast, there is no tocopherolquinone accumulation observed. It cannot be excluded that the primary tocopherol oxidation products of singlet oxygen are regenerated back to tocopherol by ascorbate or plastoquinol for example, and only when these antioxidants are consumed, tocopherol is oxidized to tocopherolquinone and other products.

We have earlier found that β-carotene is essential for D1 turnover and PSII reassembly [64]. During PSII reassembly, synthesis of new β-carotene molecules is required as they are not available from other PSII subunits for the new D1 protein. It is likely that the use of β-carotene from other subunits would destabilize their structure. The same could be also true for PQ. If the available pool of PQ is exhausted, the bound PQ would be not available for PSII reassembly. This means that the decrease in PSII activity is due to lack of PSII reassembly caused by the shortage of PQ. It is well known that the unassembled D1 protein is quickly degraded.

The results on the stimulation of PQ-C synthesis in the presence of the inhibitor under low light were unexpected and might suggest that this hydroxyl-plastoquinone, detected also in many higher plants before [28,29,32], is synthesized in a homogentisic acid-independent pathway. It is generally assumed that plastoquinone is synthesized only via homogentisic acid in plants, similarly as tocopherols. However, it was recently found [48] that mutation in *p*-hydroxyphenylpyruvate-dioxygenase in *Synechocystis* 6803, the enzyme responsible for homogentisic acid synthesis, does not affect plastoquinone synthesis. This indicates that there might be a homogentisic acid-independent plastoquinone biosynthesis pathway, at least in this cyanobacterium. Similarly, there are many cyanobacteria lacking *p*-hydroxyphenylpyruvate-dioxygenase genes and other tocopherol biosynthetic genes [47], nevertheless they are able to synthesize plastoquinone. On the other hand, cyanobacteria have also no ubiquinone and plastoquinone replaces its function in the respiratory pathway. Interestingly, in *Synechocystis* 6803 ubiquinone-like synthesis genes have been found [48] and it is possible that these genes are engaged in homogentisic acid-independent plastoquinone synthesis in cyanobacteria. We tested therefore the possibility that the ubiquinone biosynthetic pathway has a branching point after *p*-hydroxybenzoic acid towards plastoquinone. Our results show that that this is also possible in an eucaryotic organism. Our data also indicate that PQ-C is not an oxidation product of plastoquinone under high-light conditions, because its level decreases under high light, similarly as that of plastoquinone (Fig. 4). The function of PQ-C presently remains unclear in *C. reinhardtii*, although it was shown that it can function as an electron acceptor from PSII in spinach thylakoids, similarly to plastoquinone [34].

Our data suggest that plastoquinol, like tocopherol, shows photoprotective function for PSII during high-light stress by scavenging singlet oxygen generated by triplet chlorophyll in the reaction center. As a result of this reaction, plastoquinol is consumed and there must be a high resynthesis of this prenyllipid to keep its level constant to fulfill its function as a singlet oxygen quencher and hydrogen shuttle in the photosynthetic electron transport chain.

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